Isolation of Plasma Proteins from Bovine Blood by Cold Ethanol Precipitation and Anion Exchange Chromatography

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Abstract

Plasma (100.00 ml) obtained from bovine blood by centrifugation was fractionated into seven precipitates by cold ethanol precipitation. The yield (amount) of proteins in the precipitates calculated from the standard curve of BSA was 1160.83 mg, 806.57 mg, 1149.94 mg, 8.79 mg, 19.88 mg, 21.98 mg, and 13.97 mg respectively, while the total amount of protein obtained was 3180.00 mg. The precipitates were fractionated into 25 fractions each by Anion Exchange Chromatography (AEC) and the amount of protein in each fraction was obtained by Bradford protein assay. SDS-PAGE analysis was performed on the fractions with proteins and their estimated molecular weights were obtained with the aid of the molecular weight marker. The result indicated that cold ethanol precipitation and anion exchange chromatographic techniques are valuable tools for the purification of bovine blood to obtain high grade α, β and γ-fibrinogen, IgM (μ-globulin), IgG (γ -globulin), alpha (α –globulin), β-globulin (E-globulin) and albumin.

Keywords: *Plasma, chromatography, electrophoresis, albumin, fibrinogen, globulin.*

Introduction

Food and agro-allied industries usually produce large amount of wastes and where adequate disposal systems are unavailable, such wastes create environmental problems. In the meat industry the slaughtering process is the largest contributor to liquid waste. Among the liquid waste (by-product) from the slaughtering process,[1] blood is one of the most problematic due to the great amount and high pollutant load[2]. Blood effluent increases nitrogen, phosphorus and biochemical oxygen demand of any receiving water body potentially leading to eutrophication[3]. In Nigeria many slaughter houses dispose their waste directly into streams or rivers and use water from the same source to wash slaughtered meat.

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Blood comprises of plasma which is basically composed of proteins, water and a variety of salts and low molecular weight compounds[4]. Plasma is a complex body fluid that contains various proteins ranging in concentration over at least nine orders of magnitude[5]. Plasma proteins have potential economic values, it is therefore necessary to consider the recovering of proteins in the blood since treatment of this blood will help minimize environmental pollution by blood from the slaughterhouses[6]. Obviously, this is not a solution for the huge amount of blood produced, but it is a way to obtain economic benefit from a waste product[4].

Fractionation of blood proteins from blood in most cases entails separation of the plasma which is the liquid fraction of the blood from the cellular fraction by centrifugation[6]. There are various protein fractionation methods[7,8] which are based on differential solubility or differential interaction with physical parameters[7]. The methods based on differential solubility include alcohol fractionation[4,9] and the use of polyethylene glycol[8] which constitutes the major methods of differential solubility use for large scale protein purification.

Several attempts have been made to isolate proteins from matrices for instance isolation of albumin from bovine plasma by liquid chromatography and polymerisation of the isolated sample for use in immunohematology by the removal of haemoglobin, fibrinogen, globulin and nonspecific hemagglutinin[10]. Salt fractionation of plasma proteins and identification of protein fractions[11]. Coupling process for plasma protein fractionation using ethanol precipitation and ion exchange chromatography[4]. Isolation of globulin from bovine serum on Sephadex G100 gel chromatographic column[12] and isolated of albumin from bovine serum using Sephadex G100 gel chromatographic column[13]. The objective of this work is to fractionate and purify major plasma protein fractions in bovine blood by cold ethanol precipitation and anion exchange chromatographic (AEC) technique.

Experimental Methods

Materials

Thermostated stirrer, UV-Visible Spectrophotometer (Jenway 64050), crisson 2000 pH meter, peristaltic pressure pump, centrifuge apparatus (Baird and Tatlock Auto Bench), millipore glass column, fraction collectors, electrophoresis power supply (EPS 601) Amersham Pharmacia biotech, bovine serum albumin (BSA), Q-sepharose 4B, sodium citrate, absolute ethanol, sodium potassium tartarate, monosodium dihydrogen phosphate, disodium hydrogen phosphate, hydrated copper sulphate, acrylamide-bis acrylamide stock (acrylamide and bisacrylamide), Biuret reagent, Bradford reagent, coomassie brilliant blue (CBB) solution (CBB, methanol, glacial acetic acid), running gel (bis/acrylamide stock, tris HCl, SDS, ammonium persulphate, N, N, N', N'-tetramethylethylenediamine (TEMED)), sodium dodecyl sulphate (SDS), tris-glycine buffer (amino acetic acid, tris-base, SDS), tris-hydrochloric acid (tris base, HCl), 2-mercaptoethanol. All reagents used were AR grade and double distilled water was used for their preparation.

Plasma extraction from bovine blood

Bovine blood was collected from the slaughter house in Zongo, Zaria in Kaduna State, Nigeria from the vein of a six year old female bovine as it was being bled. From previous history the bovine had no record of any kind of infection. An anticoagulant, sodium citrate 2.0 % (w/v) was rapidly added at a concentration of 1.0 % (w/v) to prevent the blood from clotting. The sample was then kept on ice to maintain 4^{o}C temperature. The blood sample was centrifuged at 6000 g for 60 minutes to separate the plasma (60.0 % of the total blood volume) from the cells (40.0 % of the total blood volume). The supernatant obtained was further centrifuged for 30 minutes at 1500 g to separate the fat from the plasma. The supernatant obtained (plasma) was stored in the deep freezer at a temperature of 4^{o}C until use for maximum period of 30 h[4].

Protein precipitation from plasma

Exactly 100.00 ml of the plasma obtained was diluted to 50.0 % with cold distilled water to obtain a final volume of 200.00 ml. Ethanol (absolute) was added until the desired concentrations of 25.0 % was obtained and the pH adjusted appropriately with HCl and phosphate buffer added to keep the ionic strength of the composition constant. The mixture was allowed to stand on ice in a deep freezer for 30 minutes after which it was centrifuged for 10 minutes at 130 g[14]. The supernatant was decanted and the precipitate formed collected. The precipitation steps and conditions employed to obtain PPT I, PPT II, PPT III, PPT IV, PPT V, PPT VI and PPT VII are shown in the flow diagram in Fig. 1. The precipitates obtained were re-dissolved in 5.00 ml of 0.20 M phosphate buffer at pH 6.8[4].

Determination of amount of protein in precipitates

The amount of protein in precipitate was determined by Biuret protein assay. Standard calibration curve was prepared by reading the absorbance of 1.00-10.00 mg/ml standard concentrations of bovine serum albumin (BSA) with the UV-Visible Spectrophotometer. The obtained absorbance values were plotted against concentrations which gave a linear standard calibration curve. Sample aliquots (0.50 ml) of every test sample was placed in a test tube containing 2.50 ml biuret reagent and incubated for 30 minutes. Absorbance of the content of the test tube was measured at 540 nm with the UV-Visible Spectrophotometer. From the linear curve obtained, the concentration of protein in every precipitate was calculated according to the standard curve of BSA.

Fractionation of precipitates

Glass wool was placed at the bottom of the millipore glass column to support the resin. The resin slurry was poured into the column and allowed to settle by gravity to a bed height of 2.50 cm[15]. The column was equilibrated using 200 mM phosphate buffer solution of pH 6.8. The column was connected to a peristaltic pressure pump to exact pressure of about 200,000 N/m^2 to aid in the elution of fractions.

Sterilized syringe was used to aspirate and inject 0.50 ml of each precipitate solution obtained from ethanol precipitation into the packed column immediately after equilibrating the column. The solvents used for elution were phosphate buffer (pH 6.8), 50.00 mM, 250.00 mM, 500.00 mM and 750.00 mM sodium chloride solution prepared in phosphate buffer (pH 6.8) respectively[16].

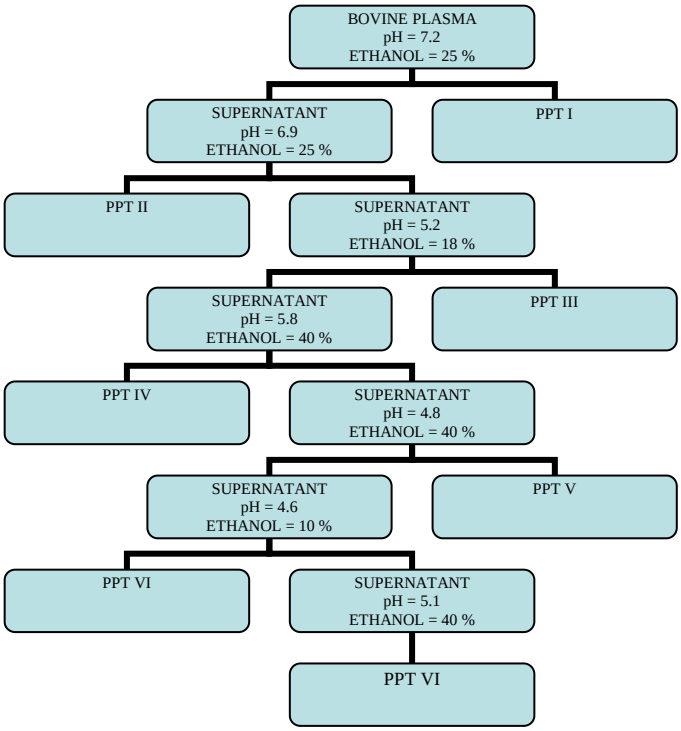

Figure 1: *Fractionation of bovine plasma by cold ethanol precipitation*

Protein components of the precipitates were separated with the equilibrated column. 25 fractions of 1.00 ml each were collected for each precipitate. Fraction 1 was the out flow from column when sample was loaded. Phosphate buffer solution (pH 6.8) was used to elute fraction 2, 3, 4; 50.00 mM sodium chloride solution was used to elute fraction 5, 6, 7, 8; 250.00 mM sodium chloride solution was used to elute fraction 9, 10, 11, 12, 13, 14, 15; 500.00 mM sodium chloride solution was used to elute fraction 16, 17, 18, 19, 20 and 750.00 mM sodium chloride solution was used to elute fraction 21, 22, 23, 24 and 25.

Determination of amount of protein in fractions from AEC

The amount of protein in fractions obtained from AEC was determined by Bradford assay. Standard concentrations (0.10 to 1.00 mg/ml) of bovine serum albumin (BSA) was prepared and placed into test tubes; the volumes were adjusted to 2.00 ml with phosphate buffer (pH 6.8); 5.00 ml of Bradford reagent was added to the content of the test tube; mixed thoroughly by inversion and the absorbance measured respectively at 595 nm with the colorimeter between 5 minutes and 60 minutes in 4.00 ml capacity cuvettes against a reagent blank prepared with 1.00 ml of the phosphate buffer (pH 6.8) and 5.00 ml of Bradford reagent. The obtained absorbance values were plotted against concentrations which gave a linear standard calibration curve. From the linear curve obtained, the amount of protein in every fraction was calculated according to the Bradford assay standard curve of bovine serum albumin (BSA)[17].

SDS-PAGE of protein fractions from AEC

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of protein fractions from AEC was carried out by treating 50.00 µl of the fractions with 50.00 µl of tris glycine buffer, one drop of mercaptoethanol, one drop of bromophenol blue and one drop of glycerol added to increase the density of the sample. This composition was placed in boiling water for 5 minutes to denature the proteins; 20.00 µl of the test samples were loaded into sample wells and electrophoresis was performed at 160 volt for one hour using the running gel. On completion of electrophoresis, gels were removed and staining procedure was carried out with coomassie brilliant blue (R-250) for 3 hours. After staining, gels were destained with 10.0 % ethanoic acid until a clear blue protein bands were observed against a clear white background and the bands photographed. The molecular weight marker was used as the standard.

Results and Discussion

Amount of proteins in precipitates

The protein concentrations in PPT I, PPT II, PPT III, PPT IV, PPT V, PPT VI and PPT VII were 24.70, 29.83, 0.08, 0.65, 1.33, 2.20 and 1.27 mg/ml, respectively. The amount in each precipitate was 1160.83 mg, 806.57 mg, 1149.94 mg, 8.79 mg, 19.88 mg, 21.98 mg and 13.97 mg, respectively, while the total amount of proteins obtained was 3181.96 mg as presented in Table 1. PPT I has the highest protein yield while PPT IV has the lowest protein yield. The total percentage of protein obtained in PPT I, PPT II and PPT III was 97.97 % while 2.03 % was obtained in PPT IV, PPT V, PPT VI and PPT VII which is an indication that most of the proteins in the plasma could be obtained in the first three steps of the fractionation procedure employed.

***Table 1**: Amount of proteins in precipitates*

Samples	Concentration in mg/ml	Total volume of PPT (cm^3)	Amount of protein in PPT (mg)	Percentage of protein in PPT
PPT I	24.6984	47.00	1160.83	36.48 %
PPT II	29.8730	27.00	806.57	25.35 %
PPT III	30.0784	37.00	1149.94	36.14 %
PPT IV	0.6508	13.50	8.79	0.28 %
PPT V	1.3254	15.00	19.88	0.62 %
PPT VI	2.1984	10.00	21.98	0.69 %
PPT VII	1.2699	11.00	13.97	0.44 %
Total amount of proteins obtained = 3181.96 mg				

Anion exchange chromatogram of precipitates

The amount of proteins recovered from AEC of PPT I, PPT II, PPT III, PPT IV, PPT V, PPT VI and PPT VII was 12.302 mg, 12.298 mg, 14.938 mg, 0.644 mg, 1.302 mg, 2.156 mg and 1.256 mg, respectively, which is 99.60 %, 82.30 %, 99.30 %, 98.90 %, 98.30 %, 98.10 % and 98.90 % recovery, respectively, as presented in Table 2. The plots of

absorbance against fractions of each precipitate are depicted in Figs 2, 3, 4, 5, 6, 7 and 8 in which the major peaks correspond to fractions with protein content.

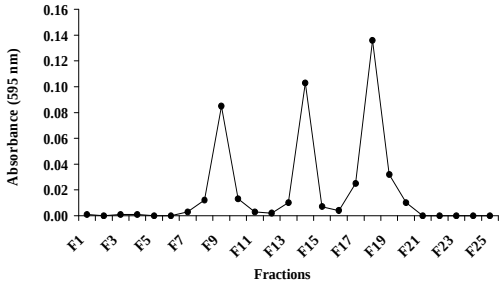

Figure 2 Anion Exchange Chromatogram of PPT I.

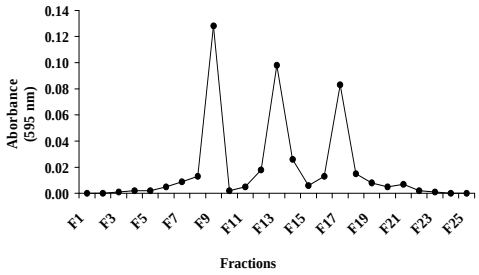

Figure 3: Anion Exchange Chromatogram of PPT II.

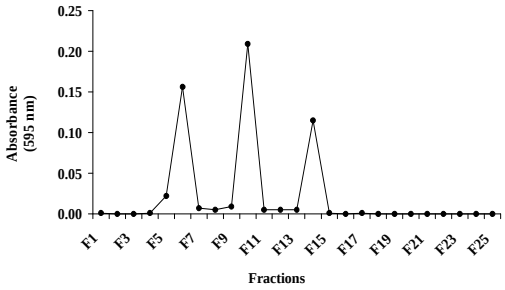

Figure 4: Anion Exchange Chromatogram of PPT III.

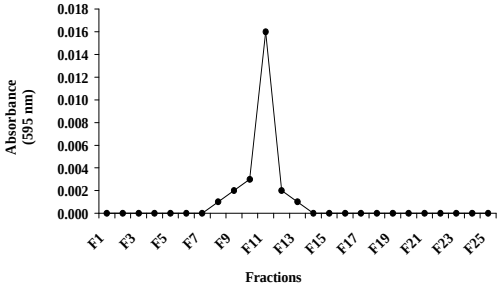

Figure 5: Anion Exchange Chromatogram of PPT IV.

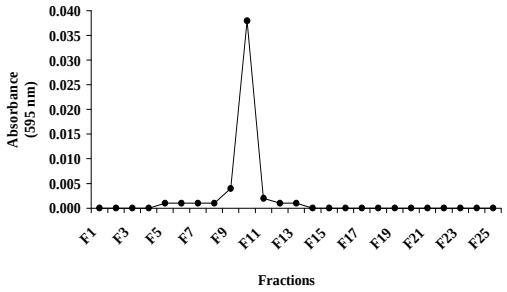

Figure 6: Anion Exchange Chromatogram of PPT V.

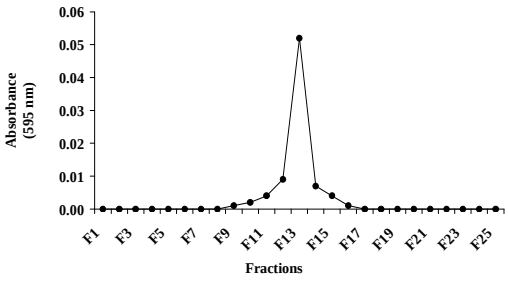

Figure 7: Anion Exchange Chromatogram of PPT VI.

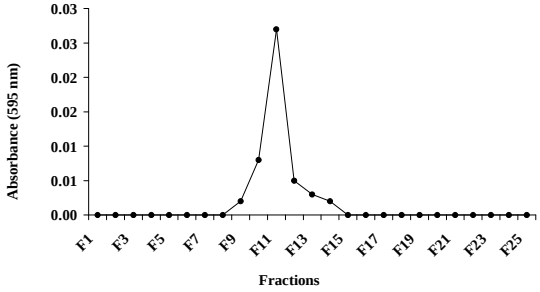

Figure 8: Anion Exchange Chromatogram of PPT VII.

Figures 2-8: *Major peaks correspond to fractions with protein content for PPT I, PPT II, PPT III, PPT IV, PPT V, PPT VI and PPT VII.*

Table 2: *Percentage recovery of protein in precipitates (PPT)*

Precipitate (PPT)	Amount loaded on column	Amount in 0.50 ml of fraction	Amount in 1.00 ml of fraction	Percentage (%) recovery
PPT I	12.349	6.151	12.302	99.6 %
PPT II	14.936	6.149	12.298	82.3 %
PPT III	15.039	7.469	14.938	99.3 %
PPT IV	0.651	0.322	0.644	98.9 %
PPT V	1.325	0.651	1.302	98.3 %
PPT VI	2.198	1.078	2.156	98.1 %
PPT VII	1.270	0.628	1.256	98.9 %

SDS-PAGE of fractions and molecular weight calculation

SDS-PAGE was performed on every fraction from AEC with conspicuous peak signifying high plasma protein component and the molecular weight estimated by comparing the migration of the proteins to standards of known weights (molecular weight marker) which is the plot of the log of the molecular weights of proteins in the standard against the relative mobility. The relative mobility (R_f) was calculated by dividing the distance the protein migrated by the total length of the gel or dye front. The molecular weights and mobility of molecular weight marker (standard) are presented in Table 3.

Table 3*: Mobility of fractions and the estimated molecular weights*

Precipitate (PPT)	Fractions	Length of gel (mm)	Mobility of fraction (mm)	Relative mobility (R_f)	Log molecular weight	Molecular weight (Da)
PPT I	F9	125	41	0.328	4.80	63,100
	F14	90	34	0.378	4.75	56,200
	F18	121	56	0.463	4.67	47,000
PPT II	F9	107	34	0.318	4.83	68,000
	F13	106	36	0.340	4.79	64,600
	F17	119	33	0.277	4.86	74,100
PPT III	F6	98	32	0.327	4.83	68,000
	F10	95	31	0.326	4.81	64,600
	F14	118	33	0.280	4.85	70,800
PPT IV	F11	106	35	0.330	4.80	63,100
PPT V	F10	104	37	0.356	4.78	60,300
PPT VI	F13	106	36	0.340	4.79	62,000
PPT VII	F13	90	32	0.356	4.78	60,300

Molecular weight of proteins in fractions

The molecular weights of plasma proteins estimated in PPT I (fractions 9, 14 and 18) were 63,100 Da, 56,200 Da and 47,000 Da, respectively, which correspond to three chains α, β and γ -fibrinogen molecules joined by disulphide bonds as depicted in plate I lane 2, 3 and 4 respectively, while lane 1 is the standard (molecular weight marker)[4]. In PPT II, the molecular weights of fractions 9, 13 and 17 were 68,000 Da, 64,600 Da and 70,100 Da which correspond to IgM (μ-globulin),[18] IgG (γ-globulin) and β-globulin (E-globulin)[4] , as depicted in plate II lane 2, 3 and 4, respectively. In PPT III, molecular weights of fractions

6, 10 and 14 were 68,000 Da, 64,600 Da and 70,800 Da which correspond to IgM (μ -globulin),[18] alpha globulin (α -globulin) and albumin as observed in plate III lane 2, 3 and 4 respectively[4]. In PPT IV, V, VI and VII, the molecular weights of fraction 11, 10, 13 and 11 were 63,100 Da, 64,300 Da, 62,000 Da and 60,300 Da which is albumin as shown in plates IV, V, VI and VII, respectively.

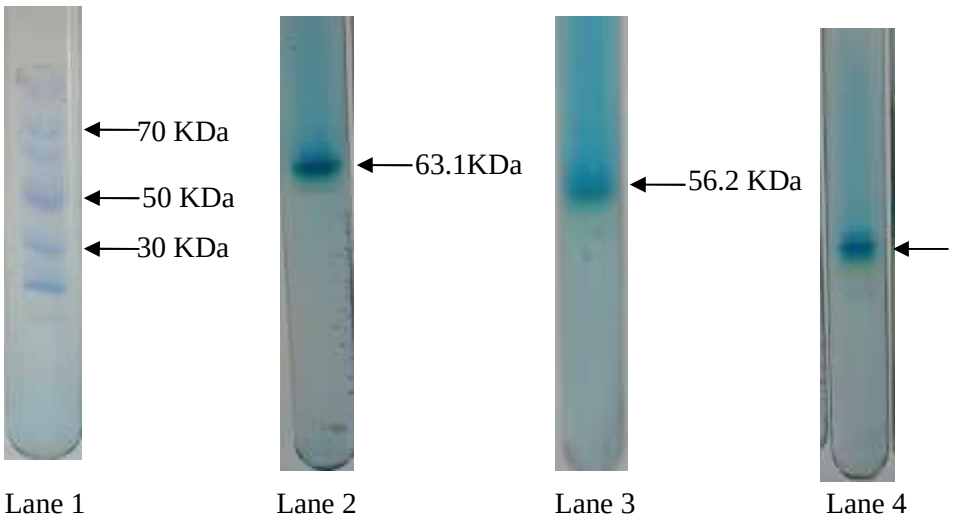

***Plate I**: Electrophoregram of fractions from PPT I*

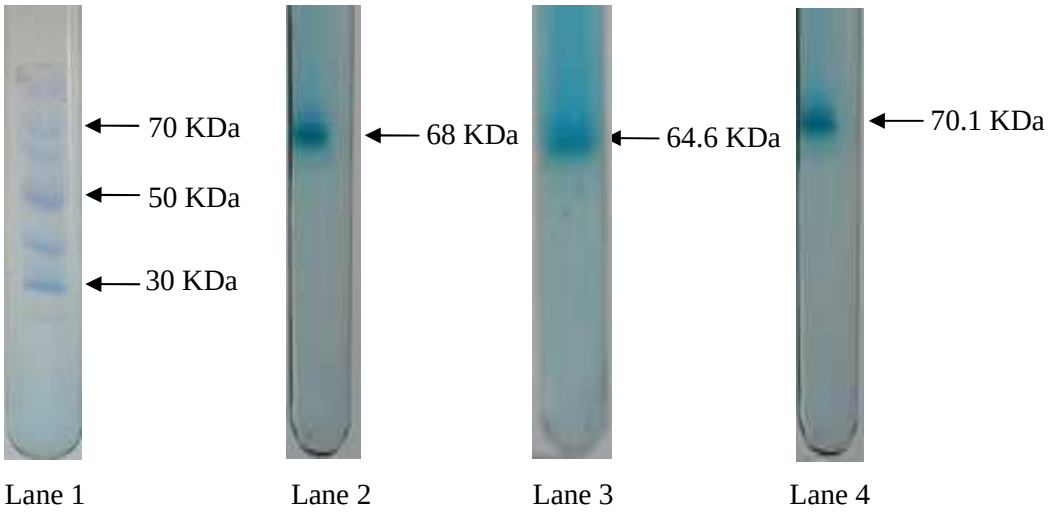

***Plate II:** Electrophoregram of fractions from PPT II*

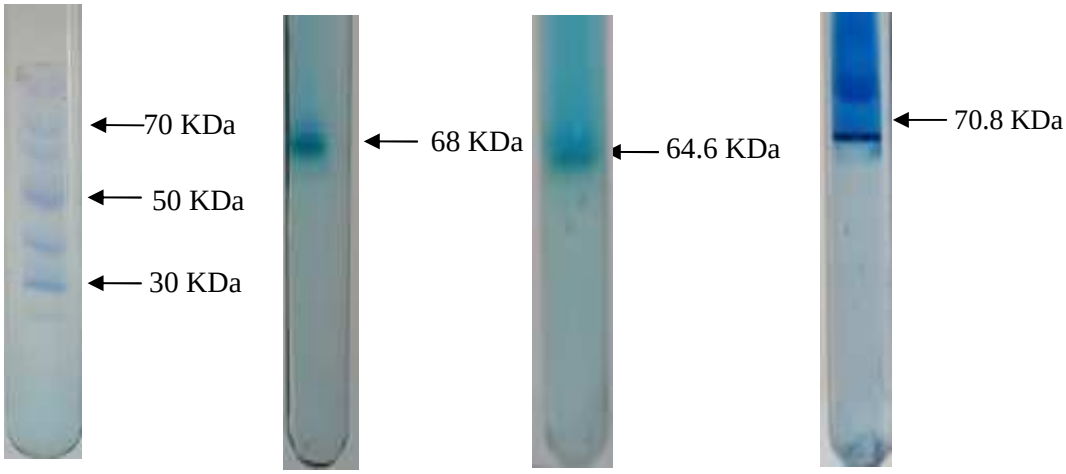

Plate III: *Electrophoregram of fractions from PPT III*

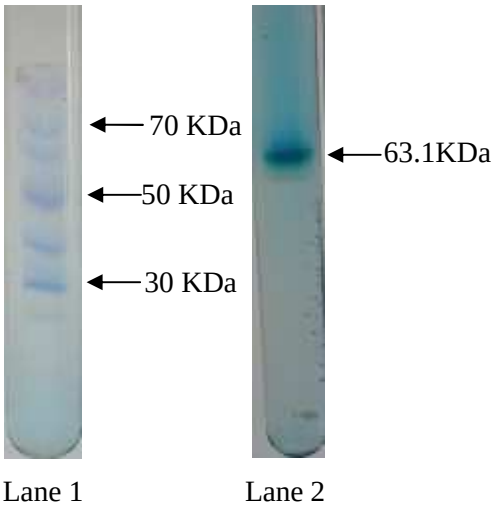

Plate IV*: Electrophoregram of fraction from PPT IV*

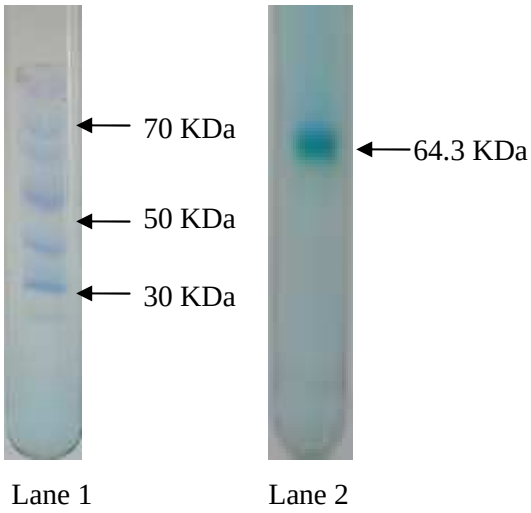

Plate V*: Electrophoregram of fraction from PPT V*

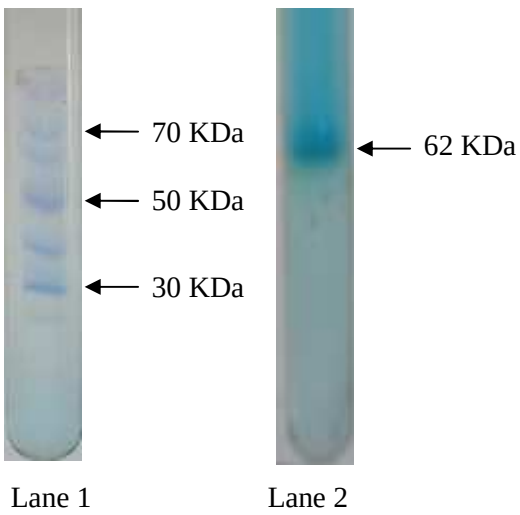

***Plate VI**: Electrophoregram of fraction from PPT VI*

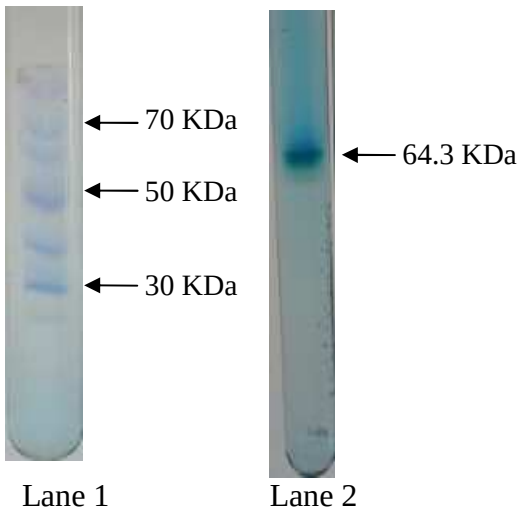

***Plate VII**: Electrophoregram of fraction from PPT VII*

Conclusions

Cold ethanol precipitation and AEC are valuable tools for the purification of bovine blood to obtain high grade α, β and γ -fibrinogen, IgM (μ-globulin), IgG (γ-globulin) alpha (α–globulin), β-globulin (E-globulin) and albumin. These proteins are extensively employed for therapeutic and diagnostic purposes. The advantage of this combined process (Cold ethanol precipitation and AEC) is that, depending on the required purity of the proteins, either fractionation with ethanol alone or both steps (fractionation and AEC) may be employed.

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